Isolation and Structural Determination of Seminal Vesicle-specific Peptides of the Terrestrial Isopod, *Armadillidium vulgare*

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During the course of purifying the androgenic gland hormone of the terrestrial isopod, *Armadillidium vulgare*, that induces post-embryonic sex differentiation, four structurally related peptides were obtained and their structures determined by a combination of microsequence and mass spectral analyses. These peptides were found to exist specifically in the seminal vesicle and vas deferens by a Western blot analysis, therefore being designated as seminal vesicle-specific peptides (SVSPs). They had essentially the same amino acid sequences but differed from one another in the truncation of several residues at the N-terminus and of one residue at the C-terminus, and in the modification of glutamine to pyroglutamate at the N-terminus. The longest peptides, SVSP-4, consisted of 60 amino acid residues with two intramolecular disulfide bridges. There is no significant homology with any other vertebrate or invertebrate peptides.

Male sexual characteristics are induced in crustaceans by a peptide hormone, the androgenic gland hormone (AGH), which is produced by androgenic glands. 1, 2 AGH seems to be peculiar to crustaceans, because insects, another big group belonging to arthropods, have a completely different sex-determining system. AGH was first extracted and partially purified from the whole male reproductive organs of the terrestrial isopod, *Armadillidium vulgare*. 3 Further purification enabled two molecular species of AGH, AGH I, and AGH II, to be obtained by an 8-step procedure and their chemical properties were characterized. 4 They were both rich in glutamic acid and/or glutamine residues and had a blocked N-terminus. Martin et al. have also recently purified two AGhs from the hypertrophied androgenic glands of intersexed animals of *A. vulgare* and found that their chemical properties were quite similar to those of AGHs from normal males already mentioned. 5 The anti-AGH antiserum has been raised against the purified AGH I fraction and, using this antiserum, AGHs were quantified by an enzyme-linked immunosorbent assay (ELISA). 6 We tried to determine the amino acid sequence of the major components in a highly purified fraction which might contain AGH I. In this report, we describe the isolation and amino acid sequences of the major components in the AGH I fraction, together with their specific existence in the seminal vesicle and vas deferens, therefore being designated as seminal vesicle-specific peptides (SVSPs).

**Materials and Methods**

*Purification of SVSPs*: The starting material for the present purification was the active fraction obtained after the 8-step purification procedure reported previously 4 and was thought to contain AGH I. Most of this fraction served as an antigen for raising antiserum as reported previously. 60 The residual material, which had been lyophilized and stored at −90°C for about 2 years, was dissolved in 20% trifluoroacetic acid (TFA) and subjected to reverse-phase high performance liquid chromatography (RP-HPLC), using a Senshu Pak RP-304 column (4.6 × 250 mm; Senshu Kagaku, Tokyo). The materials were eluted with a 40-min linear gradient of 20–40% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min, elution being monitored at both 225 and 280 nm. Peptides were determined by the absorption at 225 nm, using bovine serum albumin (BSA) as the standard.

*Enzyme-linked immunosorbent assay (ELISA)*: ELISA was performed by using an antiserum produced against the AGH-active fraction as described previously. 60

**Lysyl endopeptidase digestion**: Each sample of SVSP-1 (0.8 µg), -2 (2.4 µg), -3 (1.2 µg), and -4 (3.0 µg) was dissolved in 100 µl of a 0.1 M Tris-HCl buffer (pH 9.0) containing 2 mM urea. To this solution, lysyl endopeptidase (1 µg/µl water; Wako Pure Chemicals, Osaka) was added, and the resulting mixture was incubated at 30°C for 3 h with occasional stirring. The digestion was stopped by adding 10 µl of 1 M HCl. The digests were separated by RP-HPLC, using a Senshu Pak RP-318 column (4.6 × 250 mm; Senshu Kagaku), elution being performed by an 80-min linear gradient of 0–40% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min and monitored at 225 nm and 280 nm.

**Carboxymethylation and z-chymotrypsin digestion of SVSP-2**: SVSP-2 (1.0 µg) was dissolved in 120 µl of a 0.1 M Tris-HCl buffer (pH 7.8) containing 2 mM urea, and 10 µl of 6 mM dithiothreitol in the same buffer was added. The mixture was incubated at 45°C for 1 h with occasional shaking. To this solution, 25 µl of 6 M sodium iodoacetate in water was added, and the reaction mixture was allowed to stand at 35°C for 20 min. z-Chymotrypsin (Sigma, 1 µg/µl 1 mM HCl) was then added to the mixture, which was further incubated at 35°C for 3 h. The digestion was stopped by the addition of 10 µl of 1 M HCl. The digests were separated by RP-HPLC under the same conditions as those used for separating the lysyl endo-

* Abbreviations: AGH, androgenic gland hormone; ELISA, enzyme-linked immunosorbent assay; RP-HPLC, reverse-phase high performance liquid chromatography; SVSP, seminal vesicle specific peptide; t-Boc, tert-butyloxycarbonyl; TFA, trifluoroacetic acid.
peptidase digests.

Pyroglutamate aminopeptidase digestion of SVSP-4. SVSP-4 was digested with pyroglutamate aminopeptidase (Boehringer) as described previously. The digests were separated by RP-HPLC, using a Senshu Pak VP-304 column and an 80-min linear gradient elution by 0-40% acetonitrile in 0.05% TFA for 40 min. The recovered material was subjected to automated Edman degradation.

Amino acid sequence analysis. Intact SVSPs and their enzymatic peptide fragments were sequenced on an Applied Biosystems (ABI) model 477A protein sequencer with an on-line ABI model 130A phenylthiolyhydantoin amino acid analyzer, or on an ABI model 476A protein sequencer.

Mass spectral analyses. Mass spectra were measured on an ABI Biolon™ Biopolymer Mass Analyzer. Each sample of SVSP-2 (0.5 μg), -3 (0.4 μg), and -4 (0.5 μg) was dissolved in 10 μl of a diluted TFA solution, applied to a nitrocellulose-coated foil target, dried, washed with water, and analyzed.

Preparation of the antigen. A decapetide corresponding to the N-terminal part of SVSP-2 supplemented with a Cys residue at the C-terminus, H-Gln-Glu-Ser-Ile-Pro-Lys-Glu-Val-Leu-Cys-OH, was synthesized by the Boc method on an Applied Biosystems model 431A peptide synthesizer. Depeptidation and cleavage from the resin were accomplished according to the manufacturer’s manual. The crude peptides were purified by RP-HPLC, using a Senshu Pak ODS-H column (4.6 x 250 mm) and a 30-min linear gradient of 0-30% acetonitrile in 0.05% TFA. The amino acid sequence of the synthetic peptide was checked by sequencing. The synthetic peptide was finally conjugated with BSA, using N-hydroxysuccinimidy 3-(2-pyridylthio) propionate (SPDP, Pharmacia) as reported previously.12 to give SVSP-2(1-10)-BSA.

Production of the polyclonal antibody. Two male rabbits were immunized four times at 2-Week intervals by intraperitoneally injecting the SVSP-2(1-10)-BSA conjugate (100 μg/injection/rabbit) in Freund’s complete adjuvant. One week after the last injection, the rabbits were bled, and sera were stored at -80 °C.

Western blot analysis. The androgenic glands, testes, ovaries, seminal vesicles, vas deferens, intestines, nerves, muscles, and hepatopancreas were separately extracted with 0.1 M sodium acetate, and each extract was subjected to SDS-PAGE and a subsequent Western blot analyses as reported previously. The anti-SVSP antisera was used at a dilution of 1:200.

Results
Purification
The AGH-active materials obtained after the 8-step procedure by RP-HPLC on a Senshu Pak VP-304 column were purified again by RP-HPLC, using the same column under different conditions (Fig. 1A). The materials were separated further into at least four peaks, among which the materials from three peaks were found to be immunoreactive to the previously raised antiserum by ELISA.6 These materials were designated as seminal vesicle-specific peptide (SVSP)-1, -2, and -3 in their order of elution, because they were found to be present only in the seminal vesicle and vas deferens as described later. The amounts of these SVSPs were 1.6 μg, 8.4 μg, and 2.4 μg, respectively. All these peptides had UV absorptions at both 225 and 280 nm, although the absorbance at 280 nm was equally extremely low, suggesting that they did not contain a Trp residue (data not shown). Another molecular species of SVSP was later obtained by the same purification procedure, starting from a new lot of male reproductive organs, and was designated as SVSP-4 (Fig. 1B).

Structural analyses of SVSPs
Automated Edman degradation of each sample of intact SVSP-1 and -2 gave a single sequence. The residues up to position 44 from the N-terminal were assigned for SVSP-1, except for those at positions 11, 15, 36, 40, 41, and 43, and those up to position 26 for SVSP-2, except for the residues at positions 18 and 22 (Fig. 2). The sequence from positions 8 to 26 in SVSP-2 was identical with that of the N-terminal 19 residues in SVSP-1, indicating the structural similarity between SVSP-1 and -2. In contrast, intact SVSP-3 could not be sequenced, suggesting that the N-terminal was blocked.

The structure of SVSP-2 was first analyzed, because the yield of SVSP-2 was greatest among the three SVSPs. Lysyl endopeptidase digestion of SVSP-2 gave seven fragments, named K1–K7 (Fig. 3B), their amino acid sequences being shown in Fig. 4. K1 and K2 were easily assigned as the N-terminal and the following fragments by comparing with the N-terminal sequence of intact SVSP-2. K3 had the same sequence as K2, except for substitution of Asp by Tyr, indicating the presence of microheterogeneity in SVSP-2. K4–K7 showed essentially the same sequences. Three kinds of phenylthiolyhydantoin (PTH) derivatives of amino acid were recovered at each cycle of the Edman degradation in

Fig. 1. Purification of the Seminal Vesicle-specific Peptides (SVSPs) by RP-HPLC. (A) shows the elution profile of SVSP-1, -2, and -3, and (B) that of SVSP-4. In each case, elution was performed in a Senshu Pak VP-304 column (4.6 x 250 mm) with a 40-min gradient elution of 20-40% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min, and was monitored by the absorbance at 225 nm. Shaded peaks were immunoreactive by ELISA (see Materials and Methods). Dotted lines indicate the concentration of acetonitrile in 0.05% TFA.
the first 4 or 5 cycles and, in addition, (PTH-Cys) was clearly detected at both the first and fifth cycles. These data indicate that K4-K7 each consisted of three peptide chains connected by two disulfide bonds and that the N-terminal residue of two of the three chains was Cys, both forming Cys2. In addition, the fifth residue of one of the three chains was Cys, which was connected to another Cys residue located between positions 2 and 5 of one of the other two chains. The sequences were identified according to the N-terminal sequences of SVSP-1 and -2. The precise structural differences among the four fragments, K4-K7, remains unclear, because the C-terminal residues of two chains could not be definitely determined. However, one of the differences seems to be attributable to truncation of the C-terminal residue, Gly. Another difference seems to be due to incomplete cleavage of the Lys-Lys bond by lysyl endopeptidase. Thus, we presume that these two differences resulted in generating the four structurally close fragments, K4-K7. The connections of the three peptide chains in K4-K7 each were accomplished by aligning these peptides with the chymotryptic fragment, named C2, of S-carboxymethylated SVSP-2 (data not shown). Thus, the primary structure of SVSP-2 was established (Fig. 5) to be a mixture of 52- and 53-residue peptides with microheterogeneity at position 14.

These structures were supported by a plasma desorption (PD) mass spectral analysis (Fig. 6). Two (M + H)+ ions were observed at m/z 5979.0 and 6033.7, which coincided well with the calculated average values for 52 and 53 residues of 5979.6 and 6036.7, respectively. Judging from the spectrum, the amount of the 52-residue peptide was more than that of the 53-residue peptide.

The amino acid sequences of SVSP-1 and -3 were evaluated by comparing the elution patterns of their lysyl endopeptidase digests from RP-HPLC with that of SVSP-2 (Fig. 3). The elution patterns for SVSP-1 and -3 were similar.

**Fig. 2.** N-Terminal Amino Acid Sequences of SVSP-1 and -2. The underlined sequences in SVSP-1 and -2 are identical.

**Fig. 3.** Separation of the Lysyl Endopeptidase Digests of SVSP-1 (A), -2 (B), and -3 (C).
The lysyl endopeptidase digests were separated by RP-HPLC, using a Senso Pak VP-318 column (4.6 x 250 mm). Elution was performed by an 80-min linear gradient of 0-40% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min and was monitored at 225 nm. Dotted line indicates the concentration of acetonitrile in 0.05% TFA. The asterisked peak in (C) may be an N-terminal fragment. x indicates impurities.

**Fig. 4.** Amino Acid Sequences of the Lysyl Endopeptidase Digests of SVSP-2.

**Fig. 5.** Summary of Sequencing Data for SVSP-2. (a) N-Terminal amino acid sequence analysis, (b) sequence analyses of the lysyl endopeptidase fragments, (c) sequence analyses of chymotryptic fragments. Arrows indicate the residues identified as PTH-amino acids. SVSP-2 was a mixture of 52- and 53-residue peptides and contained a small amount of peptide (s) with the substitution of Asp by Tyr at position 14.
to that for SVSP-2, and especially all the fragments corresponding to K2, K4, K5, K6, and K7 were also present in the digests of SVSP-1 and -3, indicating that the three SVSPs shared the same sequence from position 8 to the C-terminus. The only difference between SVSP-1 and -2 was the absence of fragment K1 in SVSP-1, indicating that SVSP-1 has a sequence lacking the N-terminal 7 residues in SVSP-2. SVSP-3 also lacked the K1 fragment, but instead, a new peak (with an asterisk in Fig. 3C) was observed near K1. This fragment did not give any PTH-amino acids by Edman degradation, and is therefore regarded as the N-terminal fragment of SVSP-3. In the PD mass spectrum of SVSP-3, an (M + H)⁺ ion peak was observed at m/z 6016.9, smaller than that of AGH-2 by about 17 mass units, leading to the conclusion that SVSP-3 had been generated by pyroglutamylxation of the N-terminal Gin residue of AGH-2.

An amino-terminal amino acid sequence analysis of intact SVSP-4 did not give any PTH-amino acids, indicating the blocked N-terminus. Lysyl endopeptidase digestion of SVSP-4 and subsequent separation of the digests by RP-HPLC afforded a similar elution profile to that of SVSP-2 (data not shown). Some fragment peaks from SVSP-2, K1, K2, and one of K4–K7, were observed in the chromatogram from SVSP-4, suggesting that the structural differences between SVSP-4 and -2 were in both the N- and C-terminal sections. Digestion of intact SVSP-4 with pyroglutamate aminopeptidase generated a new peptide with a free N-terminus. Automated Edman degradation of this peptide identified the N-terminal sequence up to the 20th residue, except for the 19th residue, to be H-Tyr-Asp-Leu-Asn-Pro-Arg-Gln-Glu-Ser-Ser-Ile-Pro-Lys-Glu-Val-Leu-Glu-Asn-Xaa-Asp. As expected, the sequence from Gin⁷ to Asp³⁰ was identical with the N-terminal sequence of SVSP-2. In the PD mass spectrum of SVSP-4, (M + H)⁺, (M + 2H)²⁺, and (M + 3H)³⁺ ion peaks were observed at m/z 6907.8, 3455.6, and 2293.2, respectively. The value of 6907.8 is consistent with the value, 6907.7, calculated on the assumption that SVSP-4 would have a pyroglutamate residue at the N-terminus and a Gly residue at the C-terminus. Thus, SVSP-4 consisted of 60 amino acid residues (Fig. 7), but it remains ambiguous whether the C-terminus was amidated or not. Therefore, it can be summarized now that SVSP-1, -2, and -3 consisted of SVSP-4(15–60) and SVSP-4(15–59), SVSP-4(8–60) and SVSP-4(8–59) with additional microheterogeneity at position 19, and [pGlu⁸]SVSP-4(8–60) and [pGlu⁸]SVSP-4(8–59), respectively.

Western blot analyses

Western blot analyses were performed on various tissue extracts, using the antiserum newly raised against a synthetic decapeptide, SVSP-2(1–10). An immunoreactive band was observed at 13.5 kDa in extracts from the seminal vesicle and vas deferens (Fig. 8), but not in the extracts from any other tissues examined, including the androgenic gland. This immunoreactive material was found to be present in both the seminal vesicle tissue and its contents (Fig. 8).

Discussion

The major components in the AGH-active fraction purified after the 8-step procedure were found to be a mixture of four structurally related peptides, designated as SVSP-1 to -4, whose primary structures have been elucidated. These four peptides differed only in the N-terminal peptide chain length and modification. SVSP-4 had the longest sequence

Fig. 6. Plasma Desorption Mass Spectrum of SVSP-2.
The mass spectrum was measured on an ABI Bioion™ Biopolymer Mass Analyzer, using about 0.5 µg of SVSP-2.

Fig. 7. Amino Acid Sequence of SVSP-4.
Disulfide bridges are arranged between positions 25 and 54, and 29 and 50.

Fig. 8. Western Blot Analysis of the Extracts from Various Organs.
Extracts from various tissues of male and female A. vulgare were analyzed by Western blotting, using an anti-SVSP antiserum (1:200 dilution). Lane 1, androgenic gland (2 animal equivalents); lane 2, testis (1 animal equivalent); lane 3, seminal vesicle (0.1 animal equivalent); lane 4, vas deferens (0.2 animal equivalent); lane 5, nerve (1 animal equivalent); lane 6, intestine (0.1 animal equivalent); lane 7, hepatopancreas (0.1 animal equivalent); lane 8, muscle (0.2 animal equivalent); lane 9, ovary (1 animal equivalent); lane 10, only the tissue part of the seminal vesicle (1 animal equivalent); lane 11, seminal vesicle contents (1 animal equivalent).

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consisting of 60 amino acid residues, whose N-terminal was blocked by a pyroglutamate residue. SVSP-1, -2, and -3 were smaller than SVSP-4 by truncation of the N-terminal 14, 7, and 7 residues, respectively. Since cleavage only occurred at the carboxyl-terminal side of Arg\(^7\), Lys\(^14\), and Arg\(^9\) of SVSP-4, it is highly probable that SVSP-1, -2, and -3 were generated by partial digestion with a trypsin-like enzyme. However, it is not clear when and where this cleavage and modification occurred. Anyway, judging from the sequences of the four peptides, only SVSP-4 seems to have been a natural peptide, the other three peptides being artifacts generated during purification and/or storage.

On the other hand, the exact C-terminal structure of each peptide could not be clearly determined. The mass spectral data together with the sequencing data indicate that SVSP-2 was a mixture of two peptides different from each other only in the C-terminal residue; one terminated with Arg and the other had an additional Gly at the C-terminus. Because of the small amount of material available, we could not determine whether the carboxyl-terminus of each peptide was amidated or not.

The amino acid composition of SVSP-2 calculated from the determined sequence is very close to that of the AGH I fraction reported previously,\(^9\) indicating that SVSP-2 was really a major component of the AGH I fraction. The characteristics of a high proportion of Glu/Gln and low proportion of Pro, His, and Met in the AGH I fraction can be seen in SVSP-2.

The apparent molecular weight of 13.5 kDa of SVSP, evaluated from the mobility by SDS-PAGE, 13.5 kDa, is much bigger than the exact molecular weight of SVSP-4 of 6907 that was calculated from the sequence. This discrepancy seems to be attributable to the high proportion of acidic amino acid residues (Glu and Asp) in SVSP-4, which resulted in low mobility\(^9\) on SDS-PAGE.

A computer search did not discover any significant sequence homology with known peptides or proteins. The function of SVSPs is unclear, but considering the specific localization of SVSPs in the seminal vesicle and vas deferens, they might have some functions associated with the maintenance of sperm during storage in the seminal vesicle before mating or in the spermduct of a female after mating, or they might have some effects on the inseminated female.

We naturally expected that these four peptides were AGHs themselves or structurally and/or functionally related to AGH, when we started to analyze the amino acid sequences of these peptides. Even after obtaining the nearly complete amino acid sequences of these peptides, we still anticipated that these peptides were AGHs, partly because they were major components of the highly purified AGH-active fraction, and partly because SVSP-4 had two intramolecular disulfide bridges and a pyroglutamate residue at the N-terminus, which are often found in the peptide hormone molecules that have been characterized. However, contrary to our expectation, Western blot analyses clearly showed that no immunoreactive materials could be detected in the androgenic gland extracts, but were specifically detected in the seminal vesicle and vas deferens extracts. Based on this finding, we conclude that these peptides were not AGHs. Thus, AGH is thought to be a minor component of the AGH-active fraction purified after the 8-step procedure. Further purification is required to produce real AGH in a pure state.

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References